



Disinfection of foot-and-mouth disease and African swine fever viruses with citric acid and sodium hypochlorite on birch wood carriers

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ABSTRACT

Transboundary animal disease viruses such as foot-and-mouth disease virus (FMDV) and African swine fever virus (ASFV) are highly contagious and cause severe morbidity and mortality in livestock. Proper disinfection during an outbreak can help prevent virus spread and will shorten the time for contaminated agriculture facilities to return to food production. Wood surfaces are prevalent at these locations, but there is no standardized method for porous surface disinfection; commercial disinfectants are only certified for use on hard, nonporous surfaces. To model porous surface disinfection in the laboratory, FMDV and ASFV stocks were dried on wood coupons and exposed to citric acid or sodium hypochlorite. We found that 2% citric acid was effective at inactivating both viruses dried on a wood surface by 30 min at 22 °C. While 2000 ppm sodium hypochlorite was capable of inactivating ASFV on wood under these conditions, this chemical did not meet the 4-log disinfection threshold for FMDV. Taken together, our data supports the use of chemical disinfectants containing at least 2% citric acid for porous surface disinfection of FMDV and ASFV.

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1. Introduction

The introduction of transboundary animal disease viruses (TADV) into non-endemic countries has severe economic consequences, including the mass culling of animals and the suspension of animal trade. The importance of fomites in the spread of disease has been widely reported (reviewed in Boone and Gerba, 2007). Among the TADV, foot and mouth disease virus (FMDV), a small, nonenveloped virus, is one of the most contagious viruses known (Grubman and Baxt, 2004) and this is partly due to its remarkable ability to persist in the environment, especially on fomites (Bartley et al., 2002). African swine

fever virus (ASFV) and classical swine fever (CSFV), both highly pathogenic enveloped viruses, are also capable of spreading via fomites (Edwards, 2000; Kleiboeker, 2008).

Disinfection is crucial to prevent the spread of these highly transmissible livestock pathogens during outbreaks and to facilitate the repopulation of livestock at agricultural facilities. While there are many types of surfaces at these sites potentially contaminated with viruses, the disinfectants registered by the US Environmental Protection Agency are only recommended for hard, nonporous surfaces (USEPA, 2010). Currently, there is no standardized disinfection method for viruses on porous surfaces. Experiments describing the disinfection of TADV in suspension has been the basis for the use of disinfectants in the field, yet it is known that disinfectant efficacy is reduced when applied to dried viruses (reviewed in Springthorpe and Sattar, 2005).

Citric acid and sodium hypochlorite have been widely used for decades as disinfectants in home, health care and

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industrial settings. Sodium hypochlorite is extremely effective as a broad-spectrum microbial disinfectant (reviewed in Rutala and Weber, 1997) but due to its oxidation potential its repeated application to certain surfaces can cause corrosion (Zumelzu and Cabezas, 1996). Citric acid has also been shown to be efficacious against many microorganisms and as a natural chemical found in many food products, does not pose a significant biohazard to animals (reviewed in McDonnell, 2007). Citric acid has successfully been used against FMDV during outbreaks (Engvall and Sternberg, 2004) and is recommended by the World Organization for Animal Health for field use at a concentration of 0.2% (OIE Standards Commission, 2010).

While we have recently reported disinfection efficacy data for sodium hypochlorite and citric acid against FMDV, ASFV and CSFV dried on steel and plastic surfaces (Krug et al., 2011), there is little published information regarding the disinfection of these viruses on agriculturally relevant porous surfaces. Thus, virus disinfection on porous surfaces in the field presents a challenge due to the lack of available data and testing methodologies.

Wood construction is very common on farms thus modeling virus decontamination of wood fomites is pertinent to infection control. Other laboratories have successfully performed virus stability and disinfection assays on wood surfaces using viruses including bovine enterovirus and Newcastle disease virus (Yilmaz and Kaleta, 2003a,b), poxviruses (Hartnack et al., 2008) and avian influenza (Lombardi et al., 2008; Tiwari et al., 2006). In order to test the efficacy of disinfectants on porous surfaces, we modified the ASTM standard for virus disinfection on nonporous surfaces (ASTM, 1997) to use birch wood veneer as a porous surface model. The results presented here extend our previous nonporous surface disinfection data to the disinfection of FMDV and ASFV on a porous surface.

2. Methods

2.1. Cells and viruses

FMDV strain A24 stocks were generated in BHK-21 cells (ATCC# CCL-10). FMDV infection was identified by the presence of destructive cytopathic effects 2 or 3 days post infection. CSFV strain Brescia and the swine kidney cell line SK6 were obtained from Dr. Manuel Borca (PIADC). CSFV replication was detected by immunohistochemistry as described in Risatti et al. (2005). ASFV strain BA71/v was obtained from the PIADC virus repository and grown in Vero cells (ATCC# CCL-81). ASFV was identified by the formation of plaques after 5 to 7 days post infection. High titer virus stocks were produced as described previously (Krug et al., 2011). All virus work was conducted under biosafety level 3-Ag containment in accordance with the APHIS select agent regulations in title 9 part 121 of the code of United States federal regulations.

2.2. Disinfectants and neutralizers

1000 ppm sodium hypochlorite (Baker), mean pH $10.75 \pm$ standard deviation 0.06, was neutralized with a

2× solution of Fluid Thioglycolate Medium (FTM, Difco) All other tested concentrations of sodium hypochlorite (1500 ppm, pH 10.94 ± 0.07 and 2000 ppm, pH 11.1 ± 0.02) were neutralized with a solution containing 1× FTM and 2× Fluid Thioglycolate Broth (FTB, Fluka Chemical). 2% citric acid, pH 1.8 (Acros Organics) was neutralized with 1.25 M sodium hydroxide (Ricca Chemical). The mean pH of the neutralized solutions was 7.16 ± 0.09 . All disinfectants were diluted in 400 ppm calcium carbonate to simulate worst-case hard water conditions, and neutralizers were prepared in sterile distilled water. All disinfectants and neutralizers were made immediately before use.

2.3. Disinfection assay

This protocol is a modification of ASTM E1053: standard test method for efficacy of virucidal agents intended for inanimate environmental surfaces (ASTM, 1997) and the quantitative carrier test method described by Sattar et al. (2003). Briefly, 100 μ l of high-titer virus stock, diluted in 1× phosphate-buffered saline, was pipetted directly onto 2 cm × 2 cm × 0.1 cm coupons of autoclaved birch veneer (Rockler Woodworking) in a stainless steel base mold (Fisher Scientific #15182505C). Each virus stock was diluted to equalize the final concentration of calf serum already present in the stocks to 1.0% (v/v); excess organic load was not added in these experiments. To dry the virus, the coupons were placed in the back of a biosafety cabinet with the laminar airflow on and the lights off at ambient temperature. After the virus suspension had dried (60–90 min, depending on ambient humidity), the birch coupons were exposed to 1 ml of sodium hypochlorite or citric acid in a static temperature incubator (Incufridge, Revolutionary Science) at 22 °C. At the end of the disinfectant contact time, 1 ml of neutralizer was added directly to the disinfectant, and each birch coupon with its associated disinfectant and neutralizer fluids was transferred to a conical tube containing an equal volume (2 ml) of cell culture medium. To release virus from the wood, the liquids were mixed for 1 min at maximum speed on a vortex shaker. The liquids were then diluted and used to infect susceptible cells to determine the 50% cell culture infectious dose (CCID₅₀) titer of residual virus using the Spearman-Kärber endpoint titration method (Hierholzer and Killington, 1996). Based on assay volumes and the amount of virus added to cells, the lower limit of virus detection in this assay is 1.1 log₁₀ CCID₅₀.

2.4. Assay controls

To determine virus recovery in the absence of disinfection, birch veneer coupons with dried virus were exposed to a solution of premixed neutralizer and disinfectant for the longest contact time in each experiment, and then processed identically as the disinfection samples. This virus recovery control was used in each experiment as the 0 min contact time point, ensuring that the addition of the neutralizer to the disinfectant results in a solution that is not virucidal. No difference in virus recovery was observed when cell culture media or disinfectant/neutralizer mixtures were used, indicating the neutralization of the disinfectant was

complete (data not shown). In addition, two negative controls without virus, a surface control and a neutralization control, were included in each experiment: (1) to control for surface-induced cytotoxicity, uninoculated birch coupons were incubated with media alone and (2) to ensure effective neutralization, uninoculated birch coupons were incubated with a mixture of disinfectant and neutralizer. Both of these negative controls were processed in the same manner as the disinfection samples. Cell cultures were inoculated with these negative controls and compared to uninoculated cell cultures included on the same plate. Experiments showing visual evidence of cytotoxicity were not included in the data analysis.

3. Results

To develop a method for disinfection of viruses on porous surfaces, the ASTM standard for nonporous surface disinfection was modified to use small squares of birch wood veneer as surface coupons. A common measure of efficacy in disinfection assays is the observation of a 4-log reduction in virus recovery (USEPA, 1981). Given the $1.1 \log_{10}$ CCID₅₀/ml limit of detection in the described disinfection assay, recovering a minimum titer of $5.1 \log_{10}$ CCID₅₀/ml after drying is required to be able to measure the EPA-recommended 4-log reduction in titer by disinfectant treatment. In order to ensure enough virus could be recovered in controls to achieve this standard, FMDV, ASFV and CSFV were dried on birch coupons and virus was extracted as described in the methods. Table 1 shows the effects of drying on virus recovery from birch surfaces. FMDV exhibited the least inactivation by drying alone ($1.8 \log_{10}$ reduction) followed by ASFV ($2.5 \log_{10}$ reduction) then CSFV ($3.7 \log_{10}$ reduction). The mean recovery of FMDV ($7.0 \log_{10}$ CCID₅₀) and ASFV ($5.8 \log_{10}$ CCID₅₀) in this experiment was sufficient to use these stocks for the disinfection assay. Since CSFV mean recovery was only $3.9 \log_{10}$ CCID₅₀, this virus was not included in further wood surface disinfection experiments.

Next, assays were done to test disinfection efficacy on birch-dried FMDV and ASFV. Fig. 1 shows a time-course experiment using 2% citric acid (A) or 1000 ppm sodium hypochlorite (B) to disinfect FMDV and ASFV. Citric acid was able to reduce both ASFV and FMDV by greater than 4 \log_{10} at 20 min, however 1000 ppm sodium hypochlorite was not able to do so by 30 min. With both disinfectants, a rapid decrease in virus recovery was observed in the first 5 to 10 min. After this time, citric acid continued the

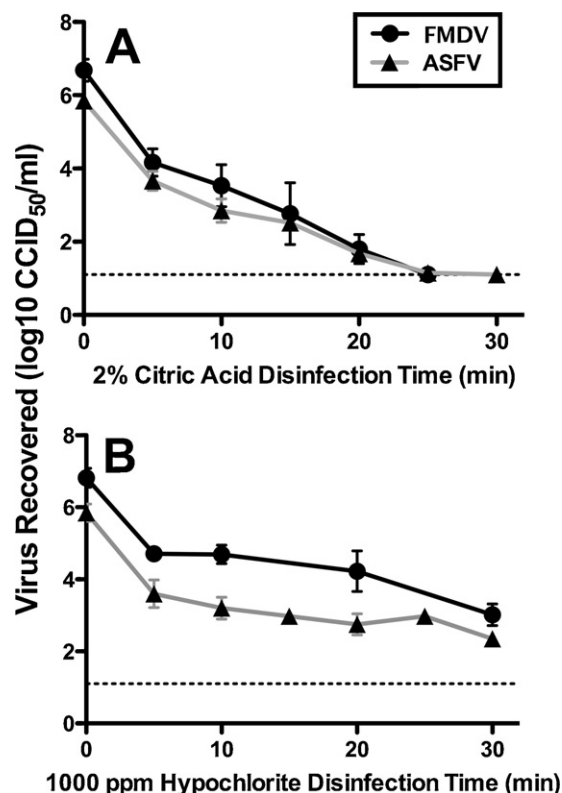


Fig. 1. Time course of TADV disinfection. FMDV or ASFV was dried on birch coupons and exposed to 2% citric acid (A) or 1000 ppm sodium hypochlorite (B). At the indicated time, the disinfectant was neutralized and the residual virus was extracted and quantified. Each point represents the mean of 3 or more experiments; error bars reflect the standard deviation. Dashed line indicates the lower limit of virus detection.

inactivation trend (Fig. 1A) and by 25 (FMDV) or 30 min (ASFV) the viruses were reduced to below the limit of detection. However, the ability for hypochlorite to extend the inactivation at the same rate was limited, as shown by the plateau between 10 and 20 min for both viruses (Fig. 1B). We interpret this difference in inactivation kinetics as both disinfectants rapidly inactivating the virus bound to the outer surface of the wood in the first 10 min but the hypochlorite was either inhibited or consumed by the wood itself in the next 20 min.

To address the possibility that increasing the hypochlorite concentration could overcome this inhibition and thereby enhance the ability to disinfect the virus in the pores of the wood, disinfection assays were performed using up to 2000 ppm sodium hypochlorite. Fig. 2 demonstrates that the increased hypochlorite concentration was able to reduce ASFV to the limit of detection by 30 min. In contrast, we observed only minor efficacy differences with the increased hypochlorite concentrations against FMDV, as treatment with 2000 ppm hypochlorite for 30 min did not result in a 4-log reduction for this virus. In a separate experiment, 2500 ppm sodium hypochlorite was not more effective at disinfecting birch-dried FMDV (data not shown).

Table 1

Recovery of TAD viruses from birch wood carriers.^a

Virus (strain)	Inoculum titer ^b	Birch recovery	log ₁₀ reduction ^c
FMDV (A24)	8.8 ± 0.63	7.0 ± 0.61	1.8
ASFV (BA71/v)	8.3 ± 0.56	5.8 ± 0.81	2.5
CSFV (Brescia)	7.6 ± 0.37	3.9 ± 0.37	3.7

^a Titers are presented as log₁₀ CCID₅₀/ml ± SD.

^b Backtiter of the diluted virus stock used to inoculate the birch coupons.

^c Determined by subtracting the birch recovery titer from the inoculum titer.

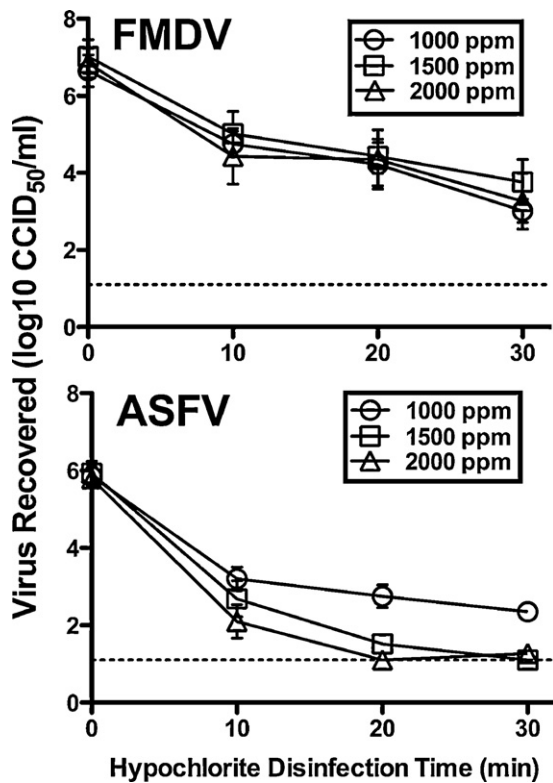


Fig. 2. Sodium hypochlorite dose–response. FMDV (top) or ASFV (bottom) was dried on birch coupons and exposed to 1000, 1500, or 2000 ppm sodium hypochlorite. At the indicated time, the disinfectant was neutralized and the residual virus was extracted and quantified. Each point represents the mean of 3 experiments; error bars reflect the standard deviation. Dashed line indicates the lower limit of virus detection.

To verify the efficacy of 2% citric acid and 2000 ppm hypochlorite for inactivation of these viruses, multiple assays with replicates were performed. The log reduction values by these chemicals are shown in Table 2. The results from the time course assays shown in Fig. 2 were confirmed as 2% citric acid was repeatedly more effective at virus disinfection than 2000 ppm hypochlorite. While most sample replicates treated with 2% citric acid had virus titers reduced to the limit of detection, this chemical was not able to completely eliminate the recovery of infectious virus, indicating that higher concentrations of disinfectant or longer contact times might be needed to achieve complete

virus inactivation. In the case of hypochlorite disinfection, we recovered infectious virus in all of the FMDV replicates (11 of 11) and almost half of the ASFV replicates (7 of 15).

4. Discussion

Here we have demonstrated a methodology to test disinfectant efficacy against viruses dried on a porous surface, based on our adaptation (Krug et al., 2011) of the ASTM standard for virus disinfection on nonporous surfaces (ASTM, 1997). Initially we tested pine wood coupons but they were found to be unusable for cell culture-based assays due to considerable cytotoxicity (data not shown). Similar cell toxicity has been previously reported for pine extracts (Mark et al., 1995). Birch wood veneer did not induce cytotoxicity in pilot studies, thus it was utilized as the porous surface carrier in the experiments presented here. Furthermore, a drying time comparison between autoclaved birch and pine veneers demonstrated no difference (data not shown), suggesting these wood types had similar performance in our experiments.

Greater than 5 log₁₀ CCID₅₀/ml of FMDV and ASFV was recovered after drying virus samples on birch coupons, allowing for the detection of at least a 4-log reduction after complete disinfection. Our results demonstrate the effectiveness of 2% citric acid for the inactivation of wood-dried FMDV and ASFV. We found that 2000 ppm sodium hypochlorite was unable to meet the 4-log inactivation threshold against FMDV. We postulate that the difference between citric acid and sodium hypochlorite may be due to consumption of the hypochlorite by the wood itself as the activity of hypochlorite can be inhibited in the presence of organic material (Terpstra et al., 2007; Weber et al., 1999). Another possibility might be that the ability of hypochlorite solutions to penetrate wood may be lower than that of acidic solutions. Indeed, it has been suggested that pH-amended hypochlorite solutions are more effective for microbial disinfection (Dychdala, 2001), however an experiment testing pH-neutral hypochlorite against wood-dried FMDV had no positive effect on efficacy in our hands (data not shown).

The highest concentration of sodium hypochlorite tested, 2000 ppm, was able to disinfect wood-dried ASFV (but not FMDV) to meet the 4-log reduction standard (Fig. 2, bottom panel), although under these disinfection conditions, approximately half of the ASFV replicates still had low levels of virus present (Table 2). The difference in inactivation by hypochlorite between FMDV and ASFV likely reflects the greater sensitivity of ASFV to hypochlorite previously reported (Krug et al., 2011). In that study, we found that sodium hypochlorite could completely disinfect ASFV at half the concentration required for complete FMDV disinfection with the same contact time on nonporous surfaces.

Disinfectant-induced cytotoxicity can introduce complications in virus disinfection assays that hamper efficacy determination. Because of the nature of cell-based assays to detect residual intact viruses, cytotoxicity can lower the detectable log reduction due to disinfectant by requiring further dilution of the disinfection samples. The US EPA guidelines for disinfectant testing take this issue into

Table 2
Log₁₀ reduction of TADV after 30 min disinfection on birch surfaces.^a

Virus	2% Citric acid	Sodium Hypochlorite	
		1000 ppm	2000 ppm
FMDV	5.22 ± 0.78 ^b n = 20 (4)	2.95 ± 0.57 n = 10 (10)	3.77 ± 0.44 n = 11 (11)
ASFV	4.72 ± 0.41 n = 13 (3)	3.75 ± 0.44 n = 9 (9)	4.43 ± 0.39 n = 15 (7)

^a Log₁₀ reduction values are determined by subtracting the mean residual virus titer from the mean recovery titer. n = total number of inoculated birch coupons disinfected (number of virus positive coupons). Each value reflects data from at least three individual experiments.

^b Contact time was 25 min for FMDV/2% citric acid.

account by accepting data demonstrating a 3-log reduction beyond the level of cytotoxicity instead of the usual 4-log reduction requirement (US EPA, 1981). It is possible, however, that a disinfectant could induce cytotoxicity but not be completely virucidal (e.g. a surfactant treatment of a non-enveloped virus). In this case, virus that potentially survived the disinfection process would not be able to replicate in the damaged cells, thereby demonstrating a false result and inflating the perceived efficacy of the disinfectant. While some disinfectant efficacy tests include a “cleanup” step utilizing column purification (ASTM, 2004), these procedures can increase disinfectant contact time and can result in a loss of virus in the resin. Therefore, in the experiments presented here, the disinfectant was completely neutralized in all assays and the virus recovery control was resuspended in a mixture of disinfectant and neutralizer to substantiate effective neutralization.

Overall, our results are in agreement with Lombardi et al. (2008) who demonstrated the failure of sodium hypochlorite and the effectiveness of citric acid for avian influenza disinfection on basswood surfaces. In that study, 1% citric acid was capable of disinfecting metal-, plastic- and wood-dried avian influenza to the limit of detection in the assay. Yilmaz and Kaleta (2003a) found that 2% formic acid was able to disinfect wood-dried picornavirus by 15 min; however, this concentration of formic acid did not inactivate reovirus or adenovirus in the same experiments.

In the current study we were not able to test CSFV disinfection on birch wood due to poor virus recovery. Other labs have also observed inefficient virus recovery from porous surfaces. We used sonication to help release CSFV from birch carriers in a manner similar to the method developed by Hartnack and colleagues (Hartnack et al., 2008) to extract dried vaccinia virus from poplar wood carriers, but that treatment failed to enhance CSFV recovery (data not shown). One reason for the low CSFV recovery in our experiments (Table 1) could be that the virus in the wood pores has been rendered noninfectious by the drying process; in this case the wood would no longer be considered a fomite since the virus is essentially inactivated. Alternatively, if residual infectious virus in the pores of the wood is trapped, the poor recovery could be due to inefficient elution as suggested by Tiwari et al. (2006). Either way, our inability to recover a sufficient titer of CSFV to perform experiments suggests that the porous surface disinfection of some enveloped viruses may require additional methodologies.

To our knowledge, this is the first report to describe the disinfection of FMDV or ASFV on a porous surface. Future investigations could adapt this method to other agriculturally relevant porous surfaces (e.g., concrete or soil) and to use infected animal fluids (e.g. vesicular fluid, saliva, and blood) as inocula to more closely simulate the virus in the field. We conclude that sodium hypochlorite-based disinfectants with 2500 ppm or less available chlorine should not be used for the disinfection of FMDV-contaminated wood surfaces. Our results are consistent with a recommendation for the use of acid-based disinfectants at pH 2 or below for the disinfection of wood surfaces contaminated with FMDV or ASFV. This report has direct implications to the selection of disinfectant products

during outbreak control and recovery from two high-consequence transboundary diseases affecting livestock populations around the world.

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